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Loss of GABAergic Signaling by AgRP Neurons to the Parabrachial Nucleus Leads to Starvation

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SUMMARY

Neurons in the arcuate nucleus that produce AgRP, NPY, and GABA (AgRP neurons) promote feeding. Ablation of AgRP neurons in adult mice results in Fos activation in postsynaptic neurons and starvation. Loss of GABA is implicated in starvation because chronic subcutaneous delivery of bretazenil (a $GABA_A$ receptor partial agonist) suppresses Fos activation and maintains feeding during ablation of AgRP neurons. Moreover, under these conditions, direct delivery of bretazenil into the parabrachial nucleus (PBN), a direct target of AgRP neurons that also relays gustatory and visceral sensory information, is sufficient to maintain feeding. Conversely, inactivation of GABA biosynthesis in the ARC or blockade of $GABA_A$ receptors in the PBN of mice promote anorexia. We suggest that activation of the PBN by AgRP neuron ablation or gastrointestinal malaise inhibits feeding. Chronic delivery of bretazenil during loss of AgRP neurons provides time to establish compensatory mechanisms that eventually allow mice to eat.

INTRODUCTION

The agouti-related peptide (AgRP)-expressing neurons and the neighboring pro-opiomelanocortin (POMC) -expressing neurons that reside in the arcuate region of the hypothalamus integrate hormonal and neurotransmitter signals to modulate appetite and metabolism and thereby help maintain energy balance (Cone, 2005; Morton et al., 2006; Saper et al., 2002). Genetic, pharmacological, and physiological data establish that enhanced melanocortin signaling by POMC neurons inhibits feeding while stimulating metabolism (Cone, 2005). AgRP neurons, which also produce neuropeptide Y (NPY) and γ-amino-butyric acid (GABA), send axons to many of the same brain regions as POMC neurons, where they antagonize the effects of melanocortin on postsynaptic cells (Broberger et al., 1998; Haskell-Luevano et al., 1999; Jacobowitz and O'Donohue, 1978; Watson et al., 1978).

The literature on regulation of body weight has emphasized the role of peptide hormones, neuropeptides, and monoamines that act on membrane receptors to activate intracellular signaling cascades (Cone, 2005; Morton et al., 2006; Saper et al., 2002), with relatively little discussion of the role of neurotransmitters that regulate ion channels (Meister, 2007), despite the fact that both $GABA_A$ and $GABA_B$ receptor agonists enhance feeding by rodents and other animals (Cooper, 2005; Duke et al., 2006; Ebenezer and Prabhaker, 2007). Research on AgRP neurons was first directed toward the role of NPY and then, after the discovery of AgRP and its coexpression with NPY, focus turned to the complementary roles of these neuropeptides in regulation of feeding behavior (Broberger and Hokfelt, 2001; Flier, 2006; Kalra et al., 1999; Shutter et al., 1997). However, inactivation of the genes encoding NPY, AgRP, or both had little effect on body weight regulation, suggesting that something else produced by these neurons was important (Erickson et al., 1996; Qian et al., 2002). Support for this idea came from experiments in which the AgRP neurons were genetically engineered to express the diphtheria toxin receptor (DTR), which allows their ablation by administration of diphtheria toxin (DT). Ablation of AgRP neurons in adult mice inhibits feeding and results in starvation within about 6 days of DT treatment (Gropp et al., 2005; Luquet et al., 2005), even in mice lacking functional *Npy* and *Agrp* genes (Phillips and Palmiter, 2008). Thus, the sudden loss of something other than NPY and AgRP promotes anorexia in this model. The starvation phenotype following AgRP neuron ablation is unaltered in the A^y genetic background, in which melanocortin signaling is blocked by ectopic production of agouti protein, indicating that starvation does not depend on activation of the melanocortin-signaling pathway (Wu et al., 2008a). Because AgRP neurons also express GABA (Cowley et al., 2001; Horvath et al., 1997), these observations led to the idea that GABA might be the critical transmitter produced by AgRP neurons. Support for this hypothesis comes from experiments in which GABA signaling by AgRP neurons was prevented by selective inactivation of the vesicular GABA transporter gene (*Vgat*); mice with constitutive inactivation of *Vgat* had a lean phenotype and were resistance to diet-induced obesity (Tong et al., 2008). This phenotype is reminiscent of the mild phenotype observed after ablation of AgRP neurons in neonatal mice (Luquet et al., 2005; Luquet et al., 2007),

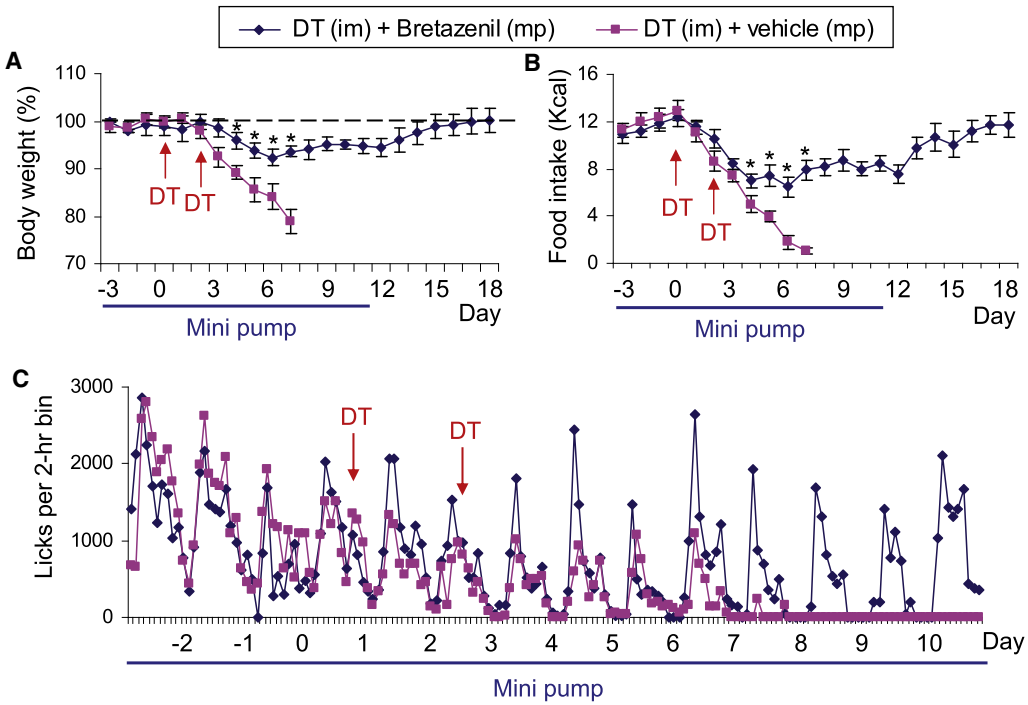

Figure 1. Chronic Administration of Bretazenil Protects against Starvation in Adult Mice after Acute Ablation of AgRP Neurons

(A) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice after subcutaneous implantation of minipump (mp) loaded with either bretazenil (n = 12) or vehicle (n = 10). DT was injected intramuscularly (im) twice as indicated by arrows. Minipumps were removed on day 11 after drug solution was depleted.

(B) Liquid diet intake by the mice described in (A).

(C) Licking activity of the mice described in (A) was plotted as average number of licks in 2 hr bins.

Error bars represent the standard error of the mean (SEM). *p < 0.01, ANOVA.

suggesting that compensation can occur when GABA production by AgRP neurons is compromised during early development.

As an alternative approach to examining the role of GABA signaling from AgRP neurons, we tested the ability of $GABA_A$ receptor agonists to maintain feeding after ablation of AgRP neurons. An extensive literature indicates that benzodiazepines can stimulate feeding and modulate taste reactivity—the stereotyped behaviors associated with different tastes (Berridge and Pecina, 1995; Cooper, 2005). Experiments in rats identified bretazenil as being particularly effective, in part, because it has less sedative activity than first-generation benzodiazepines such as diazepam. Benzodiazepines were the most effective for modulating feeding when they were delivered into the fourth ventricle. Direct injection into various hindbrain regions indicated that the parabrachial nucleus (PBN) was an important site of action in mediating taste reactivity (Higgs and Cooper, 1996). The role of GABA in the hindbrain is particularly intriguing because ablation of AgRP neurons in adult mice not only reduces the motivation to initiate meals but also blocks consumption of liquid food delivered directly into the mouth (Wu et al., 2008a), and the latter is regulated by the hindbrain (Grill and Kaplan, 2002).

We previously demonstrated robust *Fos* gene activation in numerous postsynaptic targets of AgRP neurons following ablation of AgRP neurons (Wu et al., 2008a, 2008b). These findings suggested that lack of GABA signaling from AgRP neurons could lead to hyperactivity in postsynaptic targets. The resulting dysregulation caused by lack of GABA signaling from AgRP neurons could be responsible for the blockade of both appetitive and consummatory aspects of feeding (Wu et al., 2008a). Here we show that chronic stimulation of GABAergic signaling within a specific brain region allows feeding to continue after AgRP neuron ablation and adaptations that eventually allow mice to eat without AgRP neurons.

RESULTS

Suppression of Anorexia following Ablation of AgRP Neurons by Chronic Delivery of a $GABA_A$ Receptor Agonist

Mice in which the human DTR was targeted to the *Agrp* locus (*Agrp*$^{DTR/+}$) were used for these studies. In agreement with previous results (Luquet et al., 2005; Wu et al., 2008a), administration of DT to these mice (2 intramuscular injections, 50 μg/kg, 2 days apart) results in progressive decline in food intake such that body weight falls 20% by 6–7 days, at which point the experiments are terminated for animal welfare considerations and to collect brain tissues for analysis (Figures 1A and 1B).

We showed previously that ablation of AgRP neurons by this method results in robust induction of *Fos* mRNA in most

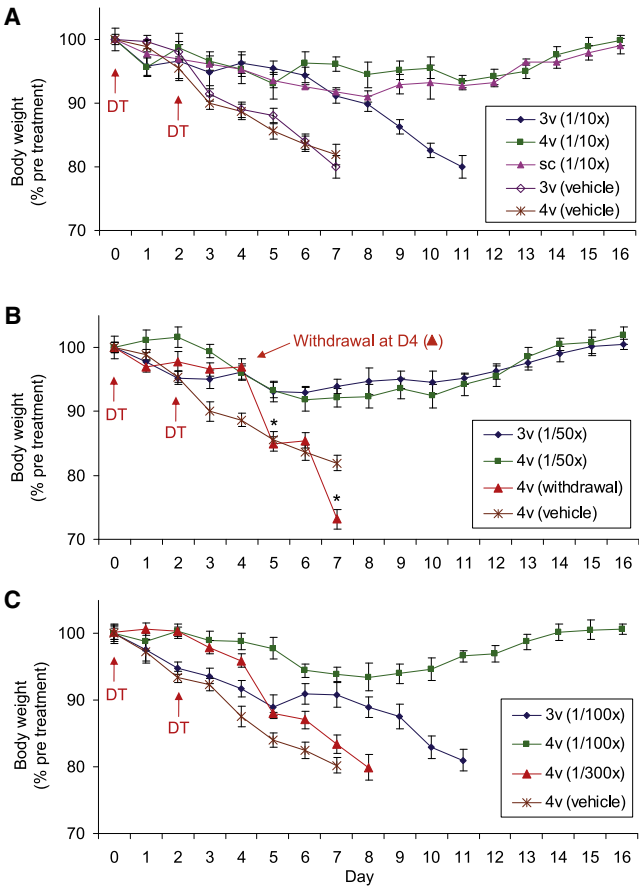

Figure 2. Minipump Delivery of Bretazenil into the Fourth Ventricle Is More Efficacious than Delivery into the Third Ventricle

(A) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice in which bretazenil (75 ng/hr; 1/10 of the dosage used in Figure 1) or vehicle was chronically administered either subcutaneously (sc) or directly into the third (3v) or fourth (4v) ventricle.

(B) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice in which bretazenil (15 ng/hr; 1/50 of the dosage used in Figure 1) or vehicle was chronically administered directly into the third or fourth ventricle. Note that abrupt withdrawal of bretazenil delivered into the fourth ventricle at day 4 resulted in more profound decline of body weight than that was shown by the AgRP-ablated mice infused with vehicle; *$p < 0.01$, ANOVA.

(C) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice in which bretazenil (7.5 or 2.5 ng/hr, 1/100 or 1/300 of the dosage used in Figure 1, respectively) or vehicle was chronically administered directly into either the third or fourth ventricle.

n = 6–8 for each group. Error bars represent the SEM.

postsynaptic targets of AgRP neurons and speculated that aberrant excitation of postsynaptic neurons could be responsible for the starvation phenotype (Wu et al., 2008a). To test this idea, Alzet minipumps were loaded with bretazenil, a $GABA_A$ receptor partial agonist, and were implanted subcutaneously to deliver 750 ng/hr for 14 days. After 3 baseline days with the bretazenil-eluting minipump, the *Agrp*$^{DTR/+}$ mice were treated with DT as before. In contrast to starvation normally observed, mice with the minipump transiently lost <10% of their body weight, and their body weight returned to normal. Food consumption by the bretazenil-treated group fell for the first 6 days after DT treatment, but then also returned to normal over the next 12 days (Figures 1A and 1B). Remarkably, feeding continued even when the minipump was removed 11 days after initiation of DT treatment (Figures 1A and 1B). Licking activity at the liquid food dispenser persisted with a typical daily rhythm in the bretazenil-treated group, whereas, in the absence of the agonist, licking ceased 7 days after DT treatment began (Figure 1C).

Bretazenil (0.2 mg/kg, i.p.) increased food consumption by wild-type mice during the first 4 hr, but 24 hr food intake was normal. Chronic delivery of bretazenil to wild-type animals for 10 days via a minipump had no effect on body weight (see Figure S1 available online). Once-daily administration of bretazenil (0.2 mg/kg, i.p.) was ineffective at preventing starvation after AgRP neuron ablation, suggesting that chronic activation of $GABA_A$ receptors is an important aspect of the rescue strategy (Figure S2).

The number of meals was maintained by mice with the bretazenil-eluting minipump after ablation, whereas it gradually declined to nothing without drug treatment (Figure S3A). The meal size (number of licks per meal) persisted at a comparable level throughout the time course in bretazenil-treated group (Figure S3B) instead of increasing as expected for food-deprived animals (Wu et al., 2008a). Ablation of AgRP neurons also prevents normal food consumption even when food is delivered directly into the mouth via an intraoral fistula, indicating that consummatory aspects of feeding are also disrupted (Wu et al., 2008a). Chronic treatment with bretazenil restored intra-oral sucrose consumption to >80% of original after ablation of AgRP neurons (Figure S3C). Thus, both consummatory and appetitive responses are rescued by bretazenil treatment after AgRP neuron ablation.

Enhanced $GABA_A$ Receptor Signaling in Hindbrain Rescues Feeding after Ablation of AgRP Neurons

To grossly determine where in the brain $GABA_A$ receptor activation is most important for maintenance of feeding, Alzet minipumps were implanted subcutaneously and connected to cannulas placed into either the third or fourth ventricles. When bretazenil was delivered at 75 ng/hr into the fourth ventricle or subcutaneously, it rescued feeding after AgRP neuron ablation; delivery of this dose into the third ventricle retarded weight loss but did not prevent starvation (Figure 2A). Mice with delivery of bretazenil into the third ventricle appeared sedated, which may have prevented adequate feeding. To minimize the sedative effects, the dose was reduced another 5 fold. Delivery of bretazenil (15 ng/hr) into either third or fourth ventricle was sufficient to prevent starvation (Figure 2B), and sedation was no longer apparent. Delivery of bretazenil into the fourth ventricle at an even lower dose (7.5 ng/hr) still maintained body weight within acceptable limits; however, delivery of this dose into the third ventricle delayed loss of body weight to 80% of original body weight (Figure 2C). A further 3-fold reduction of bretazenil delivery (2.5 ng/hr) was ineffective at preventing weight loss in the AgRP-ablated mice (Figure 2C). These results indicate that delivery of an appropriate dose of bretazenil into either the third or fourth ventricle can maintain adequate feeding, but the effect of the drug in the brainstem is marginally more effective than in the forebrain. Daily administration of bretazenil into the fourth ventricle (1 μg/day) significantly slowed the starvation phenotype

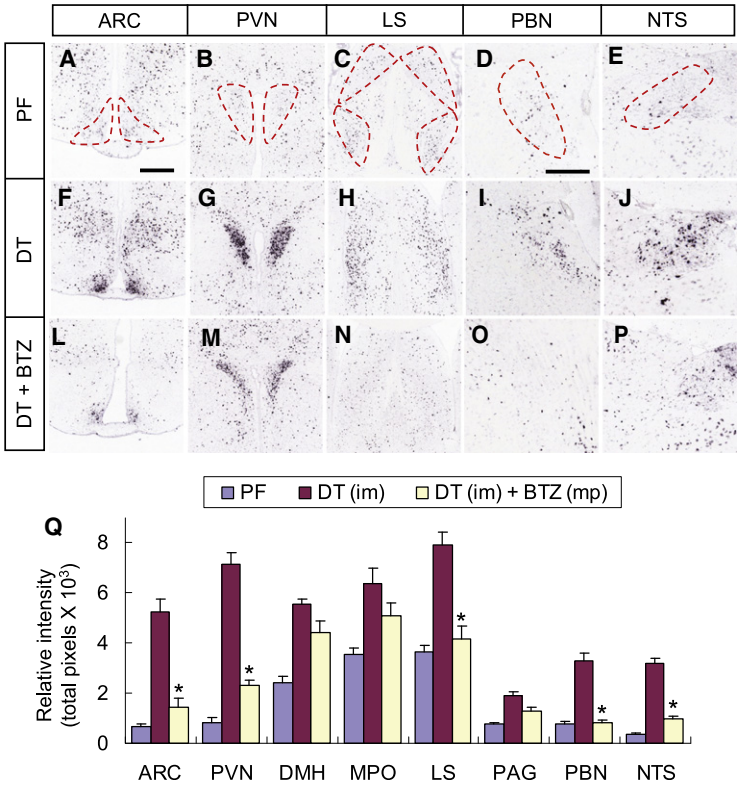

Figure 3. *Fos* Activation after AgRP Neuron Ablation Is Suppressed in Some Brain Regions by Bretazenil Treatment

(A–E) Representative pictures of *Fos* in situ hybridization in postsynaptic regions of AgRP neurons including the ARC (A), PVN (B), LS (C), PBN (D), and NTS (E), in pair-fed $Agrp^{DTR/+}$ mice after losing ~20% of their initial body weight. Postsynaptic areas of AgRP neurons are denoted by dotted lines.

(F–J) Representative pictures of *Fos* in situ hybridization in the same postsynaptic regions of AgRP neurons in DT-treated, $Agrp^{DTR/+}$ mice after losing ~20% of their initial body weight.

(L–P) Representative pictures of *Fos* in situ hybridization in the same postsynaptic regions of AgRP neurons in DT-treated, $Agrp^{DTR/+}$ mice with subcutaneous implantation of minipumps eluting bretazenil.

(Q) Quantified results for *Fos* in situ signals in selected postsynaptic regions of AgRP neurons of either pair-fed, DT-treated, or DT/bretazenil-treated, $Agrp^{DTR/+}$ mice. See Figure S6 for a complete set of images of *Fos* in situ hybridization in these regions. *$p < 0.01$ between the DT-treated group and the DT/bretazenil-treated group for each respective area, ANOVA.

n = 4–6 per group. Scale bar (in A): A–C, F–H, and L–N, 400 μm; scale bar (in D): D, E, I, J, O, and P, 400 μm. Error bars in (Q) represent the SEM.

but was unable to prevent it, again suggesting that chronic delivery of the drug is critical (Figure S4). It is noteworthy that withdrawal of the minipump delivering bretazenil at 75 ng/hr into the fourth ventricle at day 4 after initiation of DT treatment resulted in precipitous loss of body weight (Figure 2B), whereas the mice continued to feed when the minipumps were removed at day 11 (Figures 1A and 1B).

To confirm that the action of bretazenil is mediated by $GABA_A$ receptors, we blocked the effect of bretazenil with flumazenil, a $GABA_A$ receptor antagonist. Mice with minipumps delivering bretazenil (750 ng/hr, s.c.) were treated with DT as above, but then flumazenil was introduced either peripherally (10 mg/kg, i.p.) or into the third or fourth ventricle (3 μg) on a once-daily basis. Mice receiving either peripheral or the fourth ventricle delivery of the antagonist stopped feeding and rapidly lost body weight during the next 3 or 4 days, whereas mice with delivery of the flumazenil into the third ventricle survived (Figures S5A and S5B). Dispensing the same dose of flumazenil into the fourth ventricle of DT-treated, wild-type mice had no effect on food intake or body weight, as expected, since this drug only blocks the benzodiazepine site (Figures S5A and S5B). These results help establish the $GABA_A$ receptor as the critical target of bretazenil and suggest that GABA signaling in the brainstem is more important than in the forebrain for maintenance of feeding behavior in AgRP-ablated mice.

Activation of $GABA_A$ Receptors Suppresses Fos and Astrocyte Activity

Ablation of AgRP neurons leads to robust induction of *Fos* mRNA in many brain regions that are known postsynaptic targets of AgRP neurons, compared to pair-fed, control mice (Wu et al., 2008b). We hypothesized that the inhibition of feeding after ablation of AgRP neurons is due to the dysregulation of critical feeding circuits and that bretazenil acts by reducing neuronal activation. Hence, we measured *Fos* gene activation by semiautomated in situ hybridization (Lein et al., 2007). Bretazenil (minipump at 750 ng/hr, s.c.) significantly reduced *Fos* expression in 5 of the 8 brain regions that are heavily innervated by AgRP fibers (Figures 3 and S6). In the forebrain, *Fos* expression was reduced in the arcuate nucleus (ARC), paraventricular nucleus (PVN), and the lateral septum (LS), while in the hindbrain the reduction was striking in the PBN and nucleus of the solitary tract (NTS). The LS and PBN were the only anatomic positions where *Fos* expression was reduced to the level observed in pair-fed mice (summarized in Figure 3Q). Delivery of bretazenil (75 ng/hr) into the third ventricle suppressed *Fos* and astrocyte activation in the ARC and PVN but not in the PBN, whereas delivery of bretazenil into the fourth ventricle suppressed *Fos* activation in the PBN but not the ARC or PVN, indicating that appreciable amounts of the drug do not diffuse from one ventricle to another (Figure S7).

Ablation of AgRP neurons not only induces *Fos* expression in postsynaptic brain regions but also activates astrocytes and microglia in most of the same regions (Wu et al., 2008b). Release of cytokines and other molecules by activated glia may contribute to the dysregulation of feeding. We found that chronic treatment with bretazenil suppressed activation of astrocytes (revealed by GFAP immunohistochemistry) without reducing microglial activation (revealed by IBA1 immunohistochemistry) in most postsynaptic targets of AgRP neurons (Figure S8). Moreover, like the effects on *Fos* activation, the suppression of astrocyte activation was restricted to the brain regions in proximity to the ventricle where bretazenil was delivered (Figure S8).

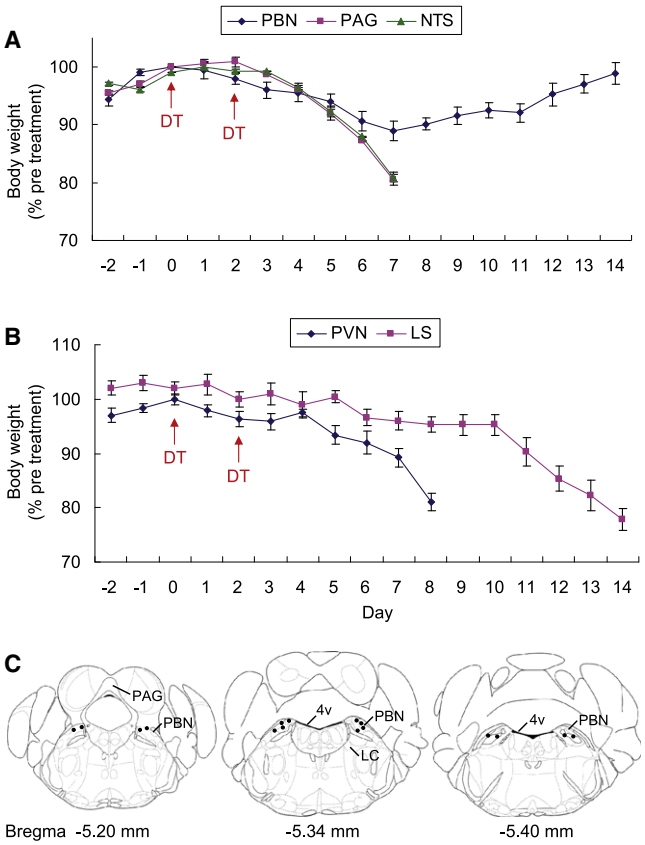

Figure 4. Administration of Bretazenil into the PBN Prevents Starvation in Mice after Ablation of AgRP Neurons

(A) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice after chronic administration of bretazenil in 3 postsynaptic areas of AgRP neurons in the hindbrain (PBN, PAG, and NTS).

(B) Percentage of initial body weight of DT-treated, *Agrp*$^{DTR/+}$ mice after chronic administration of bretazenil in 2 forebrain regions (PVN and LS) with projections from AgRP neurons.

n = 6–8 per each group. Error bars represent the SEM.

(C) Coronal illustrations of mouse hindbrain regions; the dots represent effective infusion sites in the PBN area. LC, locus ceruleus; 4v, fourth ventricle.

GABA$_A$ Receptor Signaling to the PBN Is Necessary to Prevent Anorexia

On the basis of the observation that *Fos* expression was reduced in 5 of the brain regions examined, we attempted to rescue feeding by minipump delivery of bretazenil (15 ng/hr) bilaterally into those selected brain regions. In the hindbrain, only delivery of bretazenil into the PBN achieved a full recovery of body weight following a transient loss of ~10% during the time course of treatment; whereas delivery into the PAG or NTS was ineffective (Figure 4A). In the forebrain, delivery of bretazenil to the PVN was ineffective; however, delivery into the LS prolonged survival about 1 week, but the mice eventually lost 80% of their body weight (Figure 4B). These results indicate that stimulating GABAergic signaling in the PBN is sufficient to protect against starvation in AgRP neuron-ablated mice.

The coordinates for bretazenil infusion into the PBN are shown in Figure 4C. In those cases where the cannulas were misplaced or one or both sides became clogged, anorexia was not prevented by bretazenil. The observation that delivery of bretazenil to the PAG was ineffective at preventing anorexia indicates that the effective diffusion range of bretazenil is less than 1.2 mm (the average distance from PAG to PBN). Likewise, delivery of bretazenil to the PBN suppressed *Fos* expression in the PBN but not in neighboring nuclei where *Fos* is activated after AgRP neuron ablation (data not shown).

To determine whether inhibition of GABA$_A$-receptor signaling would lead to anorexia in normal mice, bicuculline, an antagonist that binds to the GABA site in the receptor, was infused into the PBN via bilateral cannulas for 4 days, and then the pumps were disconnected. There was a dose-dependent inhibition of feeding and loss of body weight that rebounded when drug delivery ceased (Figures 5A and 5B). Thus, continual GABA signaling in the PBN is necessary to maintain feeding behavior and prevent anorexia.

To establish whether signaling from AgRP neurons to the PBN is required to maintain feeding, DT was injected into the PBN with the assumption the human DTR would be located throughout AgRP neurons, including their axons; thus, engaging DTR receptors on axonal processes would mediate cell death. Figures 5C and 5D show that *Agrp*^{DTR} mice injected bilaterally with a low dose of DT (4 ng per side) became anorexic, whereas injection of control mice with DT had no effect. Injection of the same dose of DT bilaterally into the LS did not lead to anorexia (data not shown).

AgRP Neurons in the ARC Are GABAergic

The in situ hybridization experiments included probes for *Agrp* and *Gad1* mRNA. *Gad1* encodes glutamate decarboxylase (GAD67), the major biosynthetic enzyme for GABA. Figure S9 reveals nearly complete loss of *Agrp* hybridization signal in the ARC of mice treated with DT, regardless of whether they had a bretazenil-eluting minipump. Thus, the rescue from starvation by bretazenil is not due to protection of AgRP neurons from DT-mediated ablation. Ablation of AgRP neurons with DT treatment resulted in 65% depletion of *Gad1* mRNA in the ARC, but not in the dorsomedial hypothalamus (Figures 6A–6D) indicating that AgRP neurons are the predominant population of GABAergic cells in the ARC.

Inactivation of the *Gad1* Gene in the ARC of Adult Mice Produces Anorexia

The previous results suggest that loss of GABA$_A$-receptor signaling within the PBN contributes to the severe anorexia observed after ablation of AgRP neurons. To examine whether the loss of GABA signaling from the ARC to the PBN is directly involved, we used an adeno-associated virus (AAV) strategy to inactivate GABA synthesis in the ARC. Mice with conditional *Gad1* alleles (*Gad1*$^{lox/lox}$), null alleles of *Gad2* (*Gad2*$^{-/-}$), and flox-stop Rosa26 reporter allele (*G(t)Rosa*$^{fsLacZ/fsLacZ}$) were injected bilaterally in the ARC with AAV1-CreGFP. When the Cre-expressing virus was delivered precisely to the ARC, as shown in Figure 6E (4 of 16 injected mice), food intake began to decline 5 days after viral injection and body weight fell significantly below the preinjection weight, but the mice survived at least 23 days (Figures 6H and 6I). However, when viral

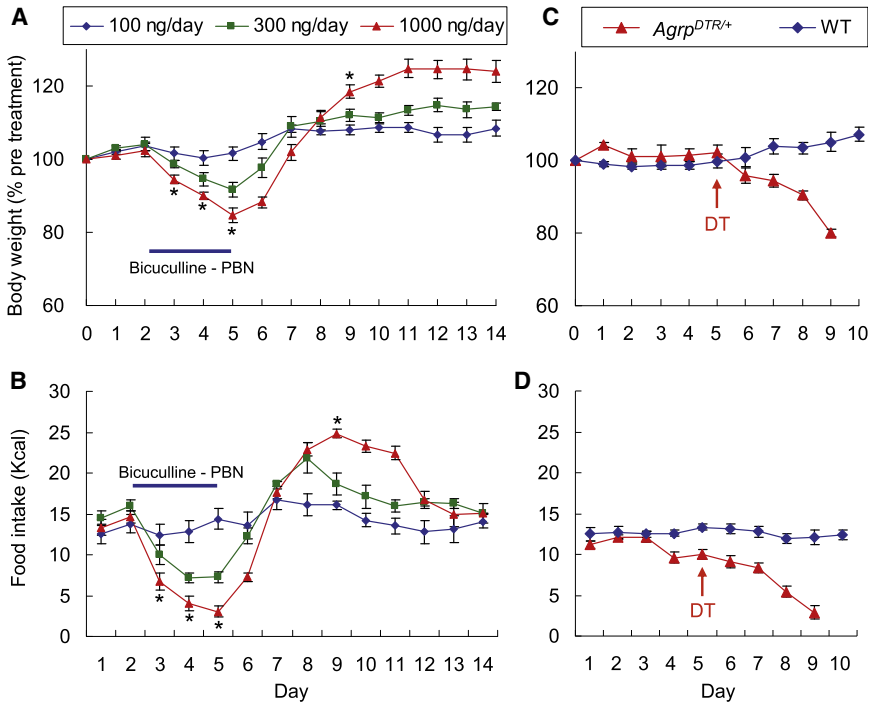

Figure 5. Inhibition of $GABA_A$ Receptors in the PBN or Disruption of the Neural Inputs from AgRP Neurons to the PBN Produces Anorexia

(A) Percentage of initial body weight of C57BL/6 wild-type mice in which bicuculline (100, 300, and 1000 ng/day) was chronically infused to the PBN area through bilateral cannulae for 4 days and then discontinued.

(B) Intake of liquid diet by the mice described in (A). *$p < 0.01$, ANOVA between the groups of 100 ng/day and 1000 ng/day. Error bars represent the SEM. n = 5–6 mice per group.

(C) Percentage of initial body weight of *Agrp*$^{DTR/+}$ and wild-type mice that received a single injection of DT into the PBN area (bilateral, 4 ng per side).

(D) Intake of liquid diet by the mice described in (C).

transduction was either unilateral in the ARC, in more dorsal regions of the hypothalamus, or undetectable (12 of 16 injected mice) there was no effect on feeding behavior (Figures 6F–6I). These results indicate that the inhibition of feeding is not an indirect consequence of viral delivery, but GABA synthesis by neurons that reside in the ARC is important for maintenance of feeding behavior.

AgRP Neurons May Directly Innervate the Fos-Positive Cells in the PBN

Broberger et al. (1998) established that the PBN is a prominent postsynaptic target of NPY fibers from hypothalamic NPY/AgRP neurons, on the basis of showing that all of the fiber staining was eliminated after destroying neurons in the ARC with monosodium glutamate treatment. To ascertain whether the Fos-positive cells in the PBN that arise after ablation of AgRP neurons are possibly direct targets of AgRP neurons, we stained sections including the PBN with antisera against Fos and NPY. This experiment was performed 4 days after the initial DT treatment, at a time when Fos was

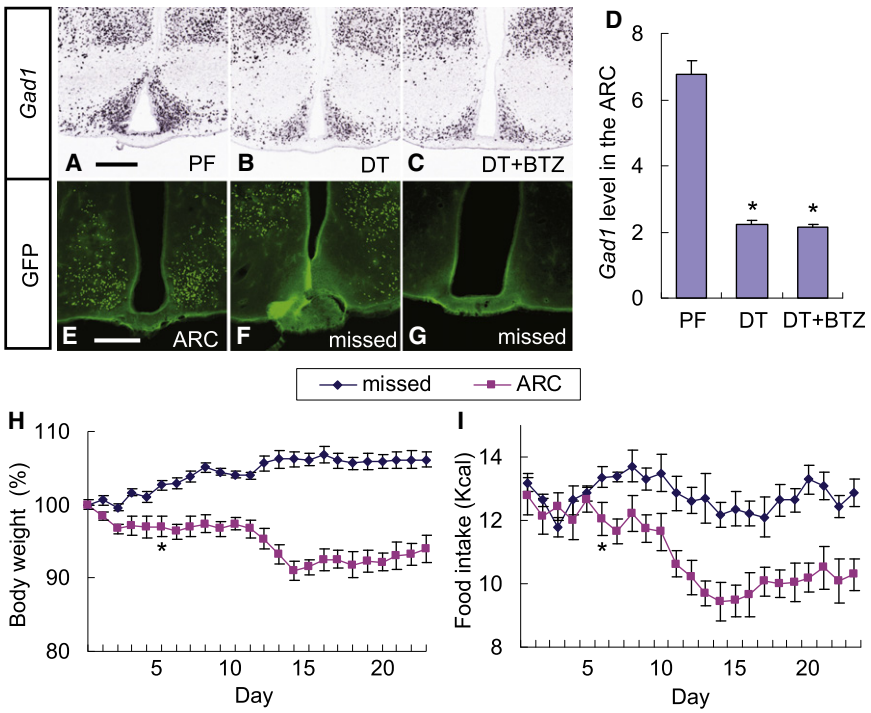

Figure 6. Loss of GABAergic Signaling by AgRP Neurons Promotes Anorexia

(A–C) Representative pictures of *Gad1* in situ hybridization in the ARC of *Agrp*$^{DTR/+}$ mice after either pair feeding (A), DT treatment (B), or DT treatment plus chronic administration of bretazenil (C).

(D) Quantified results for *Gad1* in situ signals in the ARC of the mice described in (A)–(C). *$p < 0.01$, ANOVA. Error bars represent the SEM.

n = 6 mice per each group. Scale bar (in A): A–C, 400 μm.

(E–G) Representative pictures of GFP immunostaining showing the AAV1-Cre-GFP infected neurons located either precisely in the ARC (n = 4; E) or at nonspecific surrounding areas (F) or completely absent from region (G) (n = 12) in *Gad1*$^{lox/lox}$, *Gad2*$^{-/-}$, *Rosa26*$^{fsLacZ/fsLacZ}$ mice. Scale bar (in E): E–G, 400 μm.

(H and I) Percentage change of body weight and intake of liquid diet by the mice with infection in the ARC (E) or missed (F and G). Statistical significance (*$p < 0.01$, ANOVA) was obtained starting from day 6 until end of the experiment. Error bars represent the SEM.

already activated but the AgRP/NPY neurons had not yet degenerated. Figures S10A and S10D–S10F show that Fos-positive cells are in close proximity to NPY fibers, which supports the possibility that activation of Fos may be due to direct loss of GABA signaling from AgRP neurons onto select PBN neurons. The Fos-positive cells are in the lateral segment of the PBN, in the same region where Fos is activated by peritoneal injection of LiCl, a treatment that produces gastrointestinal malaise (Figures S10B and S10C).We have also observed a large, progressive increase of GAD67 staining in fibers throughout the PBN during the 6 days after treatment of *Agrp*^{DTR} mice with DT that could reflect a compensatory increase by other GABAergic neurons (Figures S10G–S10I).

DISCUSSION

Our results establish the importance of GABA signaling by AgRP neurons onto postsynaptic $GABA_A$ receptors for maintenance of feeding behavior. Two lines of evidence support this conclusion: starvation resulting from AgRP neuron ablation can be prevented by chronic delivery of a $GABA_A$ receptor agonist, and viral-mediated inactivation of GABA biosynthesis in the ARC inhibits feeding. Furthermore, ablation of AgRP neurons in mice lacking AgRP and NPY, the other known neuromodulators made by these neurons, results in starvation; hence, loss of these neuropeptides is not responsible for the starvation phenotype (Phillips and Palmiter, 2008).

We suggest that loss of GABA signaling from AgRP neurons onto postsynaptic $GABA_A$ receptors results in unopposed excitation of postsynaptic cells as revealed by *Fos* and astrocyte activation. Our observation that inactivation of GABA synthesis in the ARC leads to anorexia suggests that direct GABA signaling by AgRP neurons to critical brain regions is important to maintain feeding after AgRP neuron ablation; however, we cannot rule out the contribution by other neurons in the ARC that produce GABA such as the POMC neurons (Hentges et al., 2004). The anorexia observed with viral inactivation of GABA synthesis was more severe than that observed by Tong et al. (2008) but still not as great as that observed with AgRP neuron ablation, presumably because only a fraction of the AgRP neurons was transduced by the virus.

We assume that chronic delivery of bretazenil during AgRP neuron ablation provides sufficient inhibitory tone to suppress the excitability of postsynaptic cells in critical feeding circuits, but suppression of cytokine production by astrocytes may also be involved. We predicted that those brain regions where *Fos* mRNA was lowered the most would reveal the most critical brain regions. We have identified two brain targets of AgRP neurons (PBN and LS) that appear to be particularly important. The most dramatic reduction of *Fos* expression was in the PBN, which is the only brain region where direct delivery of bretazenil was able to prevent starvation, although delivery of bretazenil to the LS was partially effective. Furthermore, injection of DT into the PBN (but not the LS) produced anorexia, indicating that signaling by AgRP neurons to this brain region is particularly important. Nevertheless, experiments involving chronic delivery of bretazenil to the third or fourth ventricle suggest that excessive neuronal activity in both forebrain and hindbrain may contribute to the eating disorder. The pharmacology of the agonists and antagonists used in this study indicate that GABA signaling from AgRP neurons onto $GABA_A$ receptors is sufficient to prevent starvation, but activation of $GABA_B$ receptors may also be involved. The close apposition of axonal fibers from AgRP neurons with Fos-positive neurons in the lateral PBN suggests that there may be direct GABA signaling onto the critical PBN neurons.

The PBN is a major relay for gustatory and visceral information and mediates anorexia associated with malaise induced by intraperitoneal injection of LiCl or lipopolysaccharide—treatments that are used to mimic the effects of toxic or rancid foods and bacterial infections, respectively (Rinaman and Dzmura, 2007). Lesions of PBN prevent the acquisition of conditioned taste aversion (Trifunovic and Reilly, 2002) and conditioned taste preferences (Reilly and Trifunovic, 2000). The PBN is the most responsive brain region where benzodiazepine injection promotes taste reactivity (Berridge and Pecina, 1995). Direct injections of cannabinoid or mu-opioid receptor agonists into the PBN stimulate feeding (Chaijale et al., 2008; DiPatrizio and Simansky, 2008; Wilson et al., 2003). Thus, the PBN can process both aversive and appetitive ascending visceral information to modulate feeding behavior. LiCl-induced anorexia induces robust Fos expression in the PBN and other brain regions (Andre et al., 2007; Lamprecht and Dudai, 1995; Swank and Bernstein, 1994). The Fos-positive cells in the PBN after LiCl induction are in the same region of the PBN as those induced after AgRP neuron ablation. Thus, it is likely that loss of GABAergic input to the PBN after AgRP neuron ablation activates circuits that normally promote nausea-induced anorexia. Our findings that bretazenil infusion into the PBN prevents anorexia induced by AgRP neuron ablation, while infusion of bicuculline into the PBN of normal mice promotes anorexia, are consistent with this hypothesis. The role played by the LS in feeding is more obscure. Because the LS relays signals related to sensory input and reward and projects to the lateral hypothalamus with a well-established role in feeding behavior (Bernardis and Bellinger, 1996), aberrant activity within the LS may produce anorexia via its projections to the lateral hypothalamus. Pharmacological, electrophysiological, and lesion experiments suggest a role of the LS in modulation of feeding (Scopinho et al., 2008), but the precise role of the GABAergic inputs from AgRP neurons to this brain region requires further investigation.

In addition to the consensus view that AgRP neurons oppose melanocortin signaling by POMC neurons in pathways that regulate appetite and metabolism (Cone, 2005; Morton et al., 2006; Saper et al., 2002), we propose that there are some brain regions such as the PBN where AgRP neurons have largely melanocortin-independent effects. In our view (Figure 7), ablation (or inhibition) of AgRP neurons leads to activation of POMC neurons and the melanocortin-signaling pathway, as well as a pathway involving the PBN, both of which inhibit feeding. Activation of AgRP neurons has the opposite effect. The profound anorexia that ensues from activation of the PBN—perhaps mimicking severe gastrointestinal malaise or aversive gustatory input—could mask the role of AgRP neurons in regulating appetite and metabolism by counteracting melanocortin signaling. The transient ~10% loss of body weight that still occurs when bretazenil is infused may reflect the melancortin-dependent role of

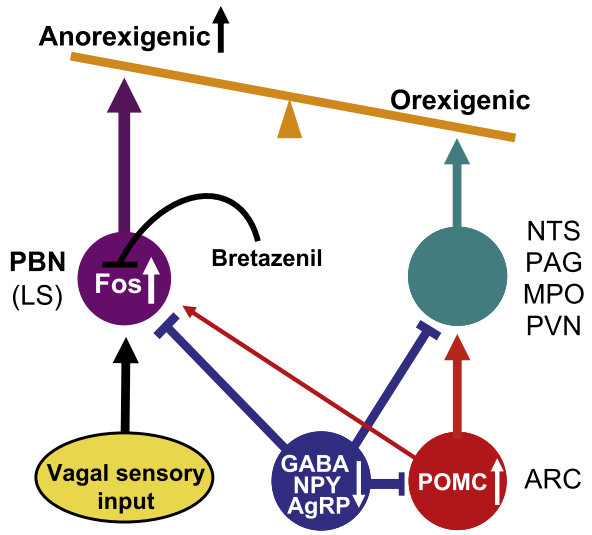

Figure 7. Diagram Illustrating How Loss of GABAergic Signaling from AgRP Neurons Leads to Starvation

AgRP and POMC neurons that reside in the ARC send axons to many of the same target areas (e.g., the PVN, MPO, PAG, NTS, and PBN). Ablation (or inhibition) of inhibitory AgRP neurons leads to anorexia by two mechanisms. First, the loss of inhibition onto POMC neurons and their postsynaptic target cells stimulates the melanocortin system, which suppresses feeding. Second, the loss of GABAergic inhibition onto neurons in the PBN (and to a lesser extent the LS) mimics the activation of these neurons in response to gastrointestinal malaise, resulting in severe anorexia, an effect that is independent of melanocortin signaling. The severe anorexia can be prevented by infusing bretazenil, a $GABA_A$ receptor partial agonist, into the PBN, which antagonizes *Fos* activation.

AgRP neurons on feeding and metabolism. If so, this effect is gradually compensated because the mice eventually regain their body weight after ~10 days, which may account for the relatively mild effect of chronic loss of GABA signaling by AgRP neurons (Tong et al., 2008).

It is noteworthy that abrupt withdrawal of bretazenil during fourth ventricle delivery results in a more precipitous decline of body weight than that achieved by ablation of AgRP neurons alone. This result mimics the loss of body weight and appetite, a common symptom of the "benzodiazepine withdrawal syndrome" seen in patients who discontinue chronic use of benzodiazepines (Pecknold, 1993). In parallel with a clinical study showing that flumazenil treatment alleviated the withdrawal syndrome (Gerra et al., 2002), our results demonstrate that bretazenil-mediated restoration of food intake was completely abolished by the fourth ventricle administration of flumazenil. On the other hand, after cessation of chronic infusion of bicuculline into the PBN of wild-type mice, their anorexia switched to prolonged hyperphagia that was inversely proportional to the dosage of bicuculline. These complementary results illustrate robust dynamics and sensitivity of a hindbrain GABA signaling network that governs appetite and food palatability.

An unexpected finding is that feeding persists in AgRP neuron-ablated mice after the bretazenil-eluting minipumps are depleted or removed. This result is reminiscent of the observation that ablation of AgRP neurons in neonatal mice does not result in starvation (Luquet et al., 2005; Phillips and Palmiter, 2008). We suggested previously (Luquet et al., 2005) that ablation of AgRP neurons in neonates has a minimal effect on feeding because adaptations can take place during the 2 to 3 weeks between ablation and weaning, when AgRP neurons mature and independent feeding becomes important (Nilsson et al., 2005). We originally thought that the adaptations might involve establishing new neuronal circuits, but the present results suggest a different explanation. We now suggest that the hyperactive postsynaptic neurons adapt by reducing the effects of excitatory inputs and/or enhancing alternative inhibitory inputs (Horvath, 2006). The increase in GAD67 staining in the PBN after AgRP neuron ablation may reflect such compensatory changes. Synaptic plasticity could also involve changes in the number of excitatory and/or inhibitory synapses as well as changes in the abundance and activity of various receptors, including ion channels and G protein-coupled receptors. Examination of the mechanisms involved will depend on more precise identification of the postsynaptic neurons with elevated Fos signal. Our results suggest that these hypothetical adaptations are incomplete and mostly ineffective 6 days after DT treatment, but can be established within 11 days with the aid of chronic $GABA_A$-receptor activation. In this view, bretazenil suppresses the excitability of postsynaptic neurons and reduces local gliosis, which, in turn, renders sufficient time and a favorable physiological setting for the adaptations to occur.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Neuron Ablation

Mice were housed in a temperature- and humidity-controlled facility with a 12 hr light/dark cycle. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. In compliance with our approved protocol, all experiments were terminated if the body weight of mice fell to 80% of their original body weight. $Agrp^{DTR/+}$ mice were generated by targeting the human diphtheria toxin receptor (heparin-binding epidermal growth factor) to the *Agrp* locus (Luquet et al., 2005). $Gad1^{lox/lox}$ mice (with loxP sites flanking exon 2) were generated as described elsewhere (Chattopadhyaya et al., 2007). They were bred with $Gad2^{-/-}$ mice (Kash et al., 1997) and $Gt(ROSA)26Sor^{fsLacZ/fsLacZ}$ mice (Soriano, 1999) to create the triple homozygotes that were used for viral injection experiments. Mice were group housed with standard chow diet (LabDiet 5053) and water provided *ad libitum* until the beginning of the experiments. To ablate AgRP neurons in adult mice, intramuscular injection of diphtheria toxin (two injections of 50 μg/kg, 2 days apart; List Biological Laboratories, Campbell, CA, USA) in 6-week-old mice was performed (Luquet et al., 2005).

Viral Injections

AAV1 virus expressing myc-nls-Cre-GFP fusion protein was kindly provided by James Allen (University of Washington). The virus was produced by transfecting HEK cells and then purified from cell extracts by pelleting through sucrose followed by banding on a CsCl gradient. Mice were anesthetized and virus was injected bilaterally (1 μl of ~10^9 particles/μl per each side) through a cannula-guided injector (31 gauge, Plastics One, Roanoke, VA, USA) just above the ARC using coordinates ± 0.5 mm (*x* axis), −1.4 mm (*y* axis), and −6 mm (*z* axis) (Paxinos and Frank, 2001). Brain samples from all mice were collected at the end of the experiment, and we proceeded with immunohistological analysis.

In Situ Hybridization

Brains were sectioned (25 μm coronal), and every eighth section was used for either Nissl staining or in situ hybridization with *Agrp*, *Fos*, or *Gad1* using an

automated procedure for hybridization and image capture. Materials and procedures concerning this high-throughput data generation process (riboprobe production, tissue processing, in situ hybridization, and image capture and processing) have been described elsewhere (Lein et al., 2007) and are available on the Allen Brain Atlas website (http://www.brain-map.org).

Drug Treatments

Alzet 14 day micro-osmotic pumps (model 1002, Durect, Cupertino, CA, USA) loaded with 100 μl of bretazenil (3 mg/ml in saline plus 10% DMSO; Sigma-Aldrich, St Louis, MO, USA) were implanted subcutaneously on the back of anesthetized mice 3 days before DT treatment. These minipumps dispense 0.25 μl/hr. Alternatively, cannulas (28 gauge, Plastics One) were placed into either the third or fourth ventricles under anesthesia, and the subcutaneous Alzet minipumps were connected to the cannulas by tubing (PE60; Stoelting, Wood Dale, IL, USA) that was threaded under the skin to help prevent the mice from dislodging it. For some experiments, the Alzet minipumps were connected to bilateral cannulas (28 gauge, Plastics One) directed to specific brain regions by using the following coordinates: the PVN, ± 0.5 mm (*x* axis), −0.8 mm (*y* axis), and −4.6 mm (*z* axis); the LS, ± 0.5 mm (*x* axis), 0.3 mm (*y* axis), and −3.8 mm (Z axis); the PBN, ± 1 mm (*x* axis), −5.3 mm (*y* axis), and −3.3 mm (*z* axis); the PAG, ± 1 mm (*x* axis), −4.75 mm (*y* axis), and −2.8 mm (Z axis); and the NTS, ± 1 mm (*x* axis), −7.4 mm (*y* axis), and −4.5 mm (*z* axis). Bretazenil used for central administration was prepared by diluting the stock solution (3 mg/ml in DMSO) in saline as indicated in the text and figure legends. For one experiment, the mice had a subcutaneous minipump loaded with bretazenil (3 mg/ml), and then flumazenil, a $GABA_A$ receptor antagonist (1.5 μl of 2 mg/ml in saline, Sigma-Aldrich), was injected either intraperitoneally or into the third or fourth ventricle via a cannula-guided injector (31 gauge, Plastics One) through a Hamilton syringe (size 25 μl, Hamilton, Reno, NV, USA). Of note, flumazenil or vehicle was injected at ~8:00 pm immediately before the mice entered active feeding phase. Bicuculline methiodide (a water-soluble form of bicuculline, Sigma-Aldrich) was prepared in saline (170 μg/ml, 50 μg/ml, and 17 μg/ml) and loaded to Alzet mini pumps for central infusion. LiCl (0.15 M in saline, J.T. Baker) was injected intraperitoneally at a dose of 20 μl/g of body weight (0.12 g/kg), then after 24 hr, the brains were collected and processed for immunohistochemistry. The patency and placement of the bilateral minipump was verified at the end of each experiment by infusing a blue dye followed by histological analysis.

Food Intake and Body Weight Measurements

For feeding assays, mice were transferred to lickometer cages (Columbus Instruments, Columbus, OH, USA) supplied with water and liquid diet (5LD-101, TestDiet, Richmond, IN, USA; 1 Kcal/ml). The mice were allowed to acclimate for 3 days before initiating each experiment and data collection. Body weight and total food intake were recorded every 24 hr. Licking activity at food and water dispensers was recorded continuously, as described elsewhere (Wu et al., 2008a). Licking data were grouped into 2 hr bins and further analyzed by using a program encoded by the Python software.

Intra-Oral Feeding Studies

Intra-oral fistulas were implanted under anesthesia at the same time when inserting minipumps loaded with bretazenil. Consumption of 0.1 M sucrose solution was measured as described elsewhere (Wu et al., 2008a).

Immunohistochemistry

Mice were sacrificed by CO_2 asphyxiation and perfused transcardially with ice-cold PBS buffer containing 4% paraformaldehyde. Brains were dissected and postfixed overnight at 4°C in the fixation buffer. Free-floating brain sections (30 μm) were washed in PBS and 0.1% Triton X-100 (PBST buffer) solution 3 × 15 min and then were blocked with 3% normal donkey serum in PBST for 2–3 hr at room temperature. Rabbit anti-GFAP (1:3000 dilution; Novus Biologicals), rabbit anti-IBA1 (1:1000 dilution; Wako Chemicals USA), guinea pig anti-NPY (1:1000 dilution; Abcam), rabbit anti-Fos (1: 1500 dilution; Millipore), and monoclonal anti-GAD67 (1:1000 dilution, Sigma) were applied to the sections for overnight incubation at 4°C, followed by 3 × 15 min rinses in PBST. Finally, sections were incubated in Cy2- or Cy3-labeled secondary antibody (1:300 dilution, Jackson Immunolaboratory) before visualization. Images were captured using a digital camera mounted on a Leica TCS SP1 confocal microscope (Leica Microsystems USA); all paired photos were obtained through the same system setting. For each group of mice, at least 8 sections from 4 different mice were analyzed.

Data Analyses

Quantification of *Fos*-positive cells was done using the NIH Image software (National Institutes of Health). Anatomical correlations of brain sections and delineation of individual nuclei were determined by comparing landmarks of Nissl staining images with those given in the stereotaxic atlas (Paxinos and Frank, 2001). From the anatomically matched sections, a region of interest of the same size was further defined. Meanwhile, an optimized threshold that can discern round *Fos*-positive nuclei from partially stained ones as well as background noise was preset for all measurement. The total number of pixels of *Fos*-positive cells inside the defined region was automatically recorded. Data sets collected from all experiments, unless otherwise stated, were analyzed by one-way ANOVA followed by Student-Newman-Keuls method for statistical significance and plotted as means ± standard error of mean (SEM). Post-hoc analysis was performed when group differences were significant by ANOVA at $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include ten figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00447-4.

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